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# Determination of diquat in biological materials by electron spin resonance spectroscopy

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**Summary.** An electron spin resonance (ESR) method already in use for the quantitative analysis of paraquat was applied to the analysis of diquat in blood, serum, urine, tissue homogenates and several drinks without purification of the samples. The diquat radical produced with ascorbic acid at alkaline pH was much more stable than that produced with the commonly used sodium dithionite. Radical decay in solutions covered with *n*-hexane was less than 5% after 60 min over a wide range of ascorbic acid concentrations. In 0.2 N NaOH solution 85% of the radicals was present even after 24 h. The limit of detection was 0.3 μg/ml and the required amount of sample was 0.1 ml. When both diquat and paraquat were present in a sample the diquat was first extracted with 1-butanol prior to the ESR measurement, because both species were converted to the radicals.

**Key words:** Diquat, determination by ESR – Paraquat

**Zusammenfassung.** Eine ESR-Methode, bereits für die quantitative Analyse von Paraquat entwickelt, wird zur Analytik von Diquat in Blut, Serum, Urin, Gewebshomogenisaten und verschiedenen Getränkeproben ohne besondere Reinigung des Materials herangezogen. Das mit Ascorbinsäure gewonnene Diquatradikal war im alkalischen Milieu stabiler als das üblicherweise eingesetzte und mit Natriumdithionit hergestellte Radikal. Der Abfall der Radikalkonzentration in n-Hexanlösungen lag bei weniger als 5% nach 60 Minuten in weiten Bereichen der Ascorbinsäurekonzentration. Beispielsweise lagen in 0,2 N-NaOH noch 85% nach 24 Stunden vor. Die Nachweisgrenze beträgt 0,3 μg/ml und das benötigte Probenvolumen 0,1 ml. Liegen sowohl Diquat als auch Paraquat in der Probe vor, so wird empfohlen, Diquat durch Extraktion mit 1-Butanol vor der ESR-Messung abzutrennen, da beide Verbindungen in Radikale überführt werden.

**Schlüsselwörter:** Diquat, Nachweis durch ESR-Methode – Paraquat

## Introduction

Diquat (1,1-ethylene, 2,2-dipyridylium) is a herbicide, and its toxicity has been investigated by many workers [1–4]. Spectrophotometric [5], gas chromatographic (GC) [6] and (HPLC) [7] methods have been used for the detection of diquat, and a new colorimetric method for the detection of diquat in water or in 1-butanol using moderate reductants has also been reported [8, 9]. The colorimetric method, however, required partial purification of the specimens to be examined (e.g. serum, blood and tissue homogenates), because they became turbid or coagulated after addition of 1-butanol [8, 9]. Therefore, an electron spin resonance (ESR) method for the detection of diquat was tested, because no pretreatment of specimens such as serum and tissue homogenates was required as described for the detection of paraquat [10, 11].

Diquat is known to become a green radical at alkaline pH by reduction with sodium dithionite, but this radical is more labile than the paraquat radical, as pointed out previously [5, 8]. The diquat radical was found to decay rapidly even if the amount of sodium dithionite was decreased to 0.02%, probably due to further reduction of the radical. This means that the sodium dithionite may be too strong to produce a stable diquat radical. Therefore, weaker reductants were examined and it was found that the diquat radical could be produced at alkaline pH using ascorbic acid.

The diquat radical is quenched with oxygen more easily than the paraquat radical, and the green color of the surface of the solution changes to yellow [8]. To avoid oxidation, the surface of the solution was covered with *n*-hexane, which does not react with diquat radicals. On the basis of these findings, a method was devised to detect diquat in blood, serum, urine, tissue homogenates and drinks without purification of the samples.

## Materials and methods

Diquat dibromide monohydrate ($C_{12}H_{12}N_2Br_2$, $H_2O$, MW 362.1; 50.87% cation) was kindly donated by Imperial Chemical Industries (Tokyo, Japan). $Mn^{2+}$ in MgO was obtained from JEOL (Tokyo, Japan) and paraquat dichloride from Sigma (St. Louis, Mo., USA). Other chemicals used were of analytical grade.

The standard diquat solution was made by dissolving 19.66 mg diquat in 10 ml 0.2 M acetate buffer, pH 4 [6]. This solution contained 1 μg/μl diquat cation. Fresh tissues used for spiking with diquat were obtained at autopsy.

*1. Radical formation.* As reported previously, 2-mercaptoethanol, *L*-cysteine, dithiothreitol and glutathione do not produce diquat radicals, but a red compound from diquat at alkaline pH [8]. It was found that ascorbic acid produced diquat radicals only at alkaline pH, whereas with sodium dithionite it was produced at pH values above 6.5. The diquat radical discharged its color when the reduced solution was vigorously shaken [5, 8]. The surface of the solution gradually became yellow due to atmospheric oxygen, even when undisturbed [8]. This change can be avoided by covering the surface of the solution. Among several organic solvents tested, *n*-hexane was found to be most suitable, because it did not mix with water and did not destroy the radical.

The pH dependence of formation of the diquat and paraquat radicals was examined as follows. Aliquots of diquat (90 μl at 10 μg/ml) were placed in small test tubes each containing 0.3 M potassium phosphate buffer at one of a range of pH values (5–13.5) and each was covered with a 0.5-cm-thick layer of n-hexane. Sodium dithionite or ascorbic acid solution (10 μl) were added in the middle of the aqueous phase using a syringe. A hematrocrit capillary was in-

serted into the reduced solution and the bottom of the capillary quickly sealed with clay. The reduced solution could be separated from air since *n*-hexane also enters at the top of the capillary.

The capillary was placed in an ESR cell together with a calibrated $Mn^{2+}$ capillary [11]. To examine the stability of the radical, ESR measurement was carried out immediately and again 60 min after mixing of the reductant. Suitable concentrations of the reductant and of NaOH were also examined using the same procedure.

The effects of interfering substances were examined as follows. Diquat (30 μg) was mixed with varying amounts of protein-denaturing agents, and the pH and the volume of the solutions were adjusted to 13.5 and 3 ml, respectively. Ascorbic acid was added to the solution at the final concentration of 0.8%. The relative amount of radicals was measured immediately and again 60 min after radical formation.

*2. Measurements of diquat in biological materials and drinks.* Tissues were homogenized in 4 volumes of distilled water using a polytron homogenizer (Kinematica, Lucerne, Switzerland). Blood and serum samples were diluted 2 × because they became viscous by alkalization. Aliquots (1 ml) of blood, serum, urine, tissue homogenates (liver, kidney, spleen, lung) and drinks (coffee, orange juice, milk, bean paste soup, wine) were each spiked with 10 μg of diquat. Then 90 μl was put in a small test tube, and 5 μl ascorbic acid solution was added from a syringe and mixed well. The solution was covered with a 5-mm-thick layer of *n*-hexane, and after 5 min, 5 μl of 4 N NaOH solution was added to the center of the aqueous phase from a syringe and mixed well. The sample was placed in a hematocrit capillary, which in turn was placed in the ESR cell together with a calibrated $Mn^{2+}$ capillary, as described before.

*3. ESR detection.* A JEOL JES-FE2XG ESR spectrometer was used with a microwave power of 5 mW and a modulation width of 2.5 gauss for diquat radicals and 0.8 gauss for paraquat radicals. The spectrometer setting was 3282 gauss, sweep range 100 gauss, sweep time 8 min and response time 0.3 s. The two calibrated $Mn^{2+}$ signals ($g = 2.0340$ and $g = 1.9810$) were measured as references of the radical amounts. When the diquat content was less than 0.5 μg/ml a sweep time of 32 min and a response time of 3 s were adopted.

## Results

Figure 1 shows an ESR signal of diquat radicals (50 μg/ml). A hyperfine structure of 13 lines was observed when measured with a modulation width of 2.5 gauss. The mean hyperfine splitting was 3.63 gauss, and the g value of the radical was 2.00354. The maximum signal height was used for quantification. The detection limit of the diquat cation was 0.3 μg/ml in 100 μl sample, as shown in Fig. 2. The resolution of hyperfine splitting of the diquat radical was lower than that of the paraquat radical [10]. Therefore, the minimum concentration of diquat that could be detected (0.3 μg/ml) was higher than that of paraquat (0.2 μg/ml).

Figures 3 and 4 indicate the pH dependence of the formation and stability of diquat and paraquat radicals, respectively, produced with sodium dithionite or ascorbic acid. Formation of diquat radicals was complete even at pH 6.5 with sodium dithionite, but decayed very quickly at this pH. For higher pH values sodium dithionite was effective in the radical formation, but the radical was unstable at any pH. Ascorbic acid produced both diquat and paraquat radicals at pH levels above 13.0, and they were stable after 1 h.

Figure 5 indicates the relationship between radical stability and the concentration of ascorbic acid in three kinds of NaOH solutions. Diquat radical was produced completely with concentrations of ascorbic acid as small as 0.2%. The decay of the radical was, however, rapid with small concentrations of ascorbic acid. When the concentration of ascorbic acid was too high, it induced lowering

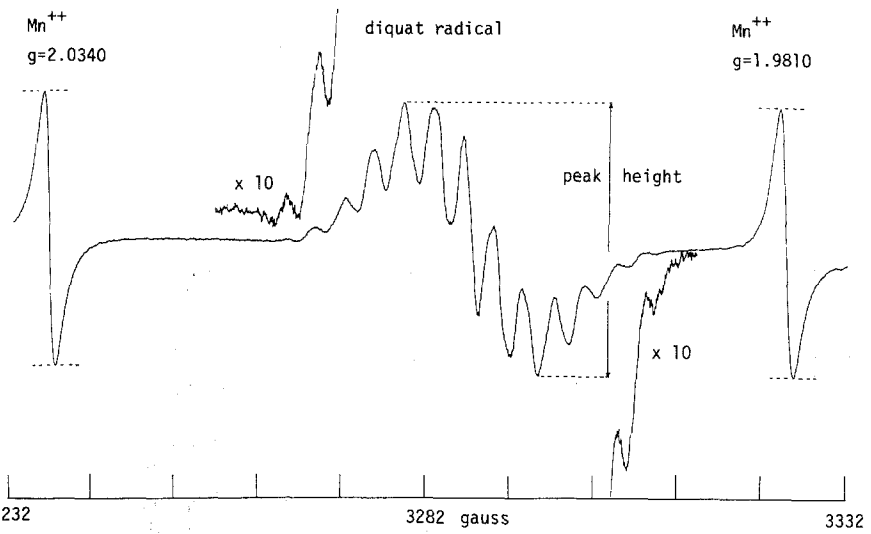


**Fig. 1.** ESR spectrum of diquat radicals (50 μg/ml). The gain setting was $5 \times 10^2$. A part of the spectrum for weaker signals is shown as *inserts* obtained using a gain setting of $5 \times 10^3$

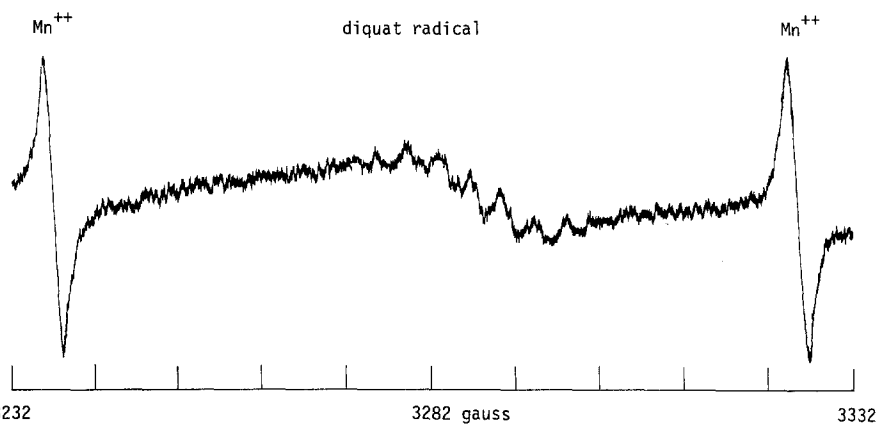


**Fig. 2.** ESR spectrum of diquat radicals at the lowest detectable concentration (0.3 μg/ml). The gain setting was $10^4$. The peak height for $Mn^{2+}$ was the same as for the diquat radical with a concentration of 1 μg/ml

of pH and, as a consequence, the formation of radicals was small at first and later attained 100%. Therefore, the concentrations of NaOH and ascorbic acid were chosen as 0.2 N and 0.4%–3.2%, respectively. Being a weaker reductant, ascorbic acid was needed in a higher concentration than was sodium dithionite, which is a strong reductant [5].

The influence of several protein-denaturing agents is shown in Fig. 6. Trichloroacetic acid and formalin strongly inhibited radical formation; perchloric acid inhibited it weakly; methanol, ethanol and acetone had no influence on radical formation.

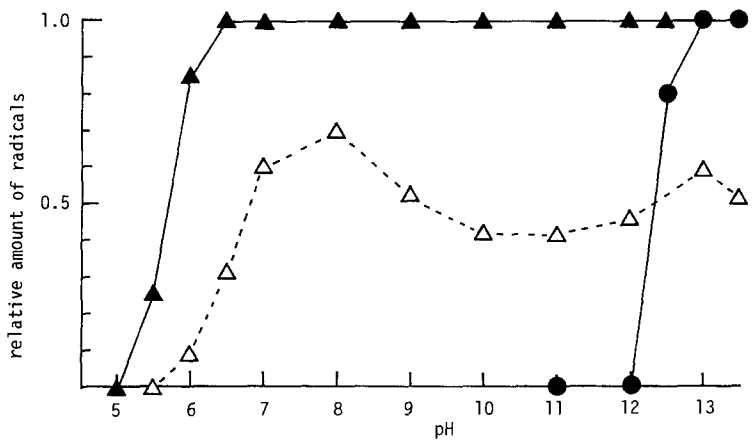


**Fig. 3.** The pH dependence of diquat radical formation in 0.3 M potassium phosphate buffer. ▲, △, Relative amounts of radicals immediately after the radical formation and after 60 min, respectively, with 0.1% sodium dithionite; ●, relative amount of radicals produced with 0.4% ascorbic acid

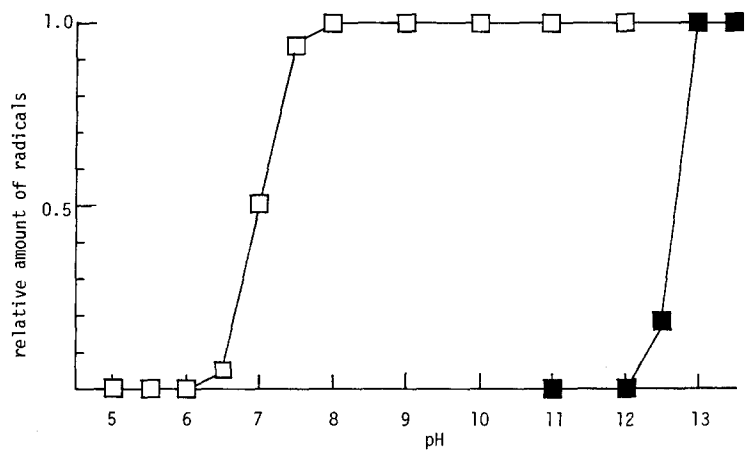


**Fig. 4.** The pH dependence of paraquat radical formation in 0.3 M potassium phosphate buffer. □ and ■ indicate the relative amounts of radicals produced with 0.1% sodium dithionite and 0.4% ascorbic acid, respectively

Figure 7 indicates absorption spectra of diquat radical produced with ascorbic acid and 0.8% ascorbic acid. The spectrum of the diquat radical was exactly the same as that of the radical produced with sodium dithionite. The absorption at around 400 nm was influenced by the existence of fresh ascorbic acid, whereas absorption at around 520 nm was influenced by oxidized ascorbic acid.

Suitable conditions for radical formation and the recoverey rate of diquat in biological materials and drinks are summarized in Table 1. As indicated, the recovery rate of diquat was more than 95% in most samples even 60 min after radical formation. The residual radicals after 24 h were found to be 85% in $H_2O$,

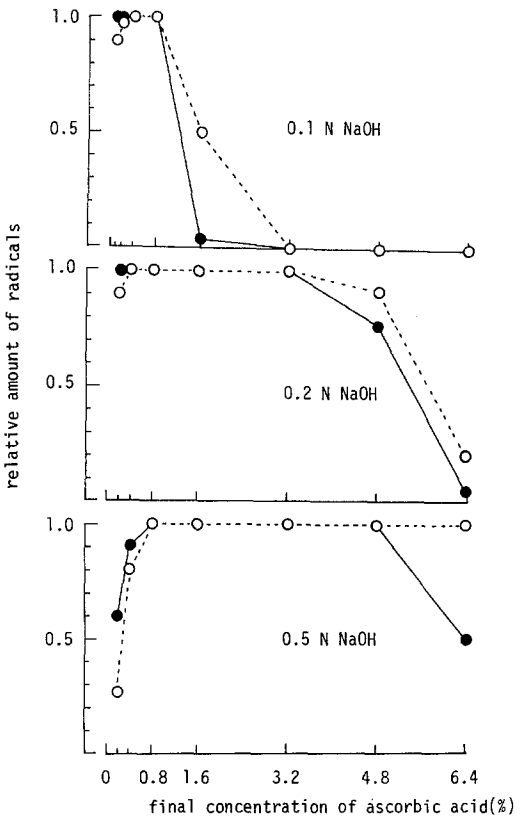


**Fig. 5.** The effect of ascorbic acid concentration on the stability of the diquat radical in 0.1, 0.2 and 0.5 N NaOH solutions. ●, ○, Relative amounts of radicals immediately after the radical formation and after 60 min, respectively

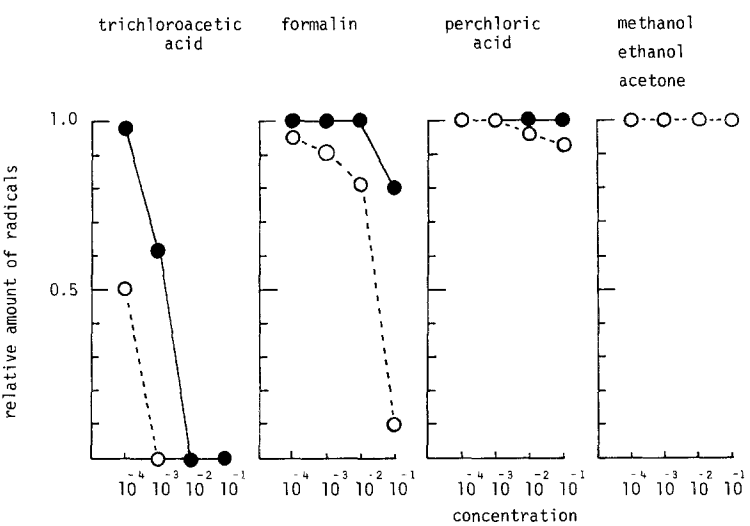


**Fig. 6.** Effect of protein-denaturing agents on the formation and the stability of the diquat radical. ●, ○, Relative amounts of radicals immediately after the radical formation and after 60 min, respectively

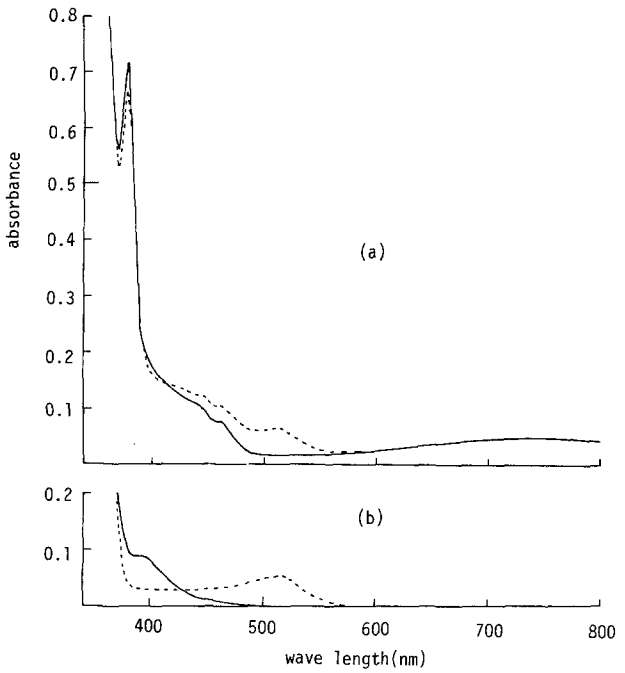


**Fig. 7a, b.** Absorption spectra of the diquat radical. **a** Absorption spectra of 3 μg/ml diquat radicals produced with 0.8% ascorbic acid in 0.2 N NaOH solution —— immediately, ---- 60 min after the radical formation. **b** Absorption specta of 0.8% ascorbic acid in 0.2 N NaOH solution —— immediately, ---- 60 min after the addition of NaOH

**Table 1.** Recovery rates of diquat from various samples

| Material | Concentration of ascorbic acid | Relative amounts of radicals | |
|---|---|---|---|
| | | 0 min | 60 min |
| Blood diluted twice | 3.2% | 100% | 100% |
| | 1.6 | 90 | 90 |
| | 0.8 | 20 | 20 |
| | 0.4 | 0 | 0 |
| Serum diluted twice | 1.6 | 100 | 100 |
| | 0.8 | 100 | 85 |
| Urine | 0.4 | 100 | 95 |
| Liver homogenate | 1.6 | 100 | 100 |
| | 0.8 | 100 | 95 |
| Kidney homogenate | 1.6 | 100 | 100 |
| | 0.8 | 100 | 90 |
| Spleen homogenate | 1.6 | 100 | 100 |
| | 0.8 | 100 | 85 |
| Lung homogenate | 1.6 | 100 | 98 |
| | 0.8 | 95 | 80 |
| Coffee | 0.8 | 100 | 95 |
| Orange juice | 1.6 | 100 | 94 |
| Milk | 1.6 | 100 | 90 |
| Bean paste soup | 1.6 | 100 | 90 |
| Wine | 1.6 | 100 | 85 |

35% in blood diluted twice and 20% in liver homogenates, respectively. Oxyhemoglobin greatly interfered with radical formation. A solution of 0.2% ascorbic acid was enough for 100% reduction of diquat in aqueous solution, whereas the reduction of diquat in blood diluted twice was 0, 20%, 90% and 100% with 0.4%, 0.8%, 1.6% and 3.2% ascorbic acid, respectively. Among the samples examined blood required the highest concentration of ascorbic acid for reduction of diquat.

## Discussion

As discussed in previous papers [10, 11], the ESR method is suitable for identification and quantification of radicals, because the ESR signal is specific to radicals. Furthermore, the ESR method is simple because removal of other substances is unnecessary when these do not produce radicals. The radical of ascorbic acid itself can be generated in acidic solution with the aid of peroxidase [12] and is detected by a flow-ESR method, because the radical is quite unstable. In the present method, ascorbic acid in 0.2 N NaOH did not produce any radicals whose signals overlapped with the signal of the diquat radical, as shown in Figs. 1 and 2.

Radicals are more or less unstable chemical species. In the present study, not only the efficiency of radical formation, but also the stability of the radical produced is quite an important factor for the quantitative detection of diquat. The diquat radical produced with ascorbic acid was stable at alkaline pH in the presence of high concentrations of ascorbic acid, as mentioned before. In addition, fading of the color of the radical proceeded from the surface of the solution, so that it is necessary to protect the sample from oxidation. Addition of *n*-hexane is an easy and appropriate way to prevent exposure to air, because *n*-hexane does not mix with water or destroy the diquat radical.

The hyperfine structure of the ESR signals of diquat radicals became obscured as the viscosity of the solution increased, due to immobilization of the radical in solution. Blood and serum were almost coagulated after alkalization with NaOH, so that these samples were diluted by 50% before addition of NaOH.

Paraquat, a well-known herbicide, is often used mixed with diquat [1–7]. As shown in Fig. 4, paraquat radical was also produced under the same conditions as diquat radical. When both were present, the ESR signals of the two radicals overlapped. When 2-mercaptoethanol was added to the mixture of diquat and paraquat, a red compound was produced from diquat only and could be extracted with 1-butanol, the paraquat remaining in the aqueous phase [9]. After separation of diquat, extraction should be repeated prior to the addition of ascorbic acid for measurement of diquat by the ESR method, because diquat radicals cannot be produced in 1-butanol [9].

As seen in Fig. 7, the radical produced with ascorbic acid showed the same absorption spectrum as that produced with sodium dithionite [5]. For this reason, ascorbic acid can be used as a reductant of a color test of diquat for a short time after mixing. Ascorbic acid, however, gradually turns to a brown compound in alkaline solution, as seen in Fig. 7. Therefore, ascorbic acid is inapplicable in color testing of diquat in dilute solution in the later stage. On the other hand, an ordinary spectrophotometric measurement is always applicable for quantification of diquat, because the reference solution also contains the same amount of ascorbic acid which turns to a brown compound.

Ascorbic acid should be added before alkalization when samples containing blood are used. The diquat radical in blood could not be detected when NaOH was first added followed by ascorbic acid, whereas 80% or 100% of radicals could be detected in serum or aqueous solution, respectively, using the same procedure, perhaps because reduction of the oxyhemoglobin at acidic pH is essential for the formation of diquat radicals.

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